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Award Number: DAMD17-02-1-0315

TITLE: Targeting Breast Cancer Vasculature

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REPORT DATE: March 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 074-0188*



| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | March 2003 | Annual (1 Mar 02 - 28 Feb 03) |

**4. TITLE AND SUBTITLE**
Targeting Breast Cancer Vasculature

**5. FUNDING NUMBERS**
DAMD17-02-1-0315

**6. AUTHOR(S):**
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**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**
Original contains color plates: All DTIC reproductions will be in black and white.

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**12b. DISTRIBUTION CODE**

**13. Abstract** ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
The main problems with current cancer therapies, including those for breast cancer, are that they are only partially effective and highly toxic. We work on a strategy that enhances the efficacy of anti-tumor therapies, while simultaneously decreasing the side effects. Our target is the vasculature of tumors. Tumor cells depend on blood supply and the tumor vasculature is accessible through the blood stream. An added advantage is that the vasculature is composed of normal cells, which are unlikely to develop resistance to treatments.

We identify tumor-specific vascular markers by screening phage-displayed peptide libraries in mice bearing breast cancer xenografts or endogenous transgenic breast cancers. When the libraries are intravenously injected into the mice, the phage that have specific affinity for tumor vasculature home to the tumors. These peptides can then be used to carry drugs and other therapeutics into tumors.

During the past year, we have identified (a) a tumor-homing peptide that binds to a collagen that is over-expressed in breast cancer vasculature and (b) peptides that home to lymphatic vessels in tumors. The homing peptides that target tumor lymphatics represent an important extension of the vascular targeting technology. Destroying the lymphatics and tumor cells in and around them with targeted drugs may be particularly useful in preventing metastasis.

| 14. SUBJECT TERMS: | 15. NUMBER OF PAGES |
|---|---|
| breast cancer | 21 |
| | 16. PRICE CODE |

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

# Targeting Breast Cancer Vasculature

## Progress Report 3/2003

## TABLE OF CONTENTS

# DOD Breast Cancer Innovator Award
# Progress Report Yr 02

## INTRODUCTION

Toxic side effects limit the usefulness of many of the existing anti-cancer drugs. If it were possible to selectively target the drug into the tumor tissue, the efficacy of anti-tumor therapies could be enhanced while simultaneously decreasing the side effects. We are working on a targeting strategy that aims at physically concentrating therapeutic agents in tumor tissue by making use of the unique features of tumor vasculature.

Directing a therapy at the tumor vasculature has advantages: First, the vasculature is available for the therapeutic agent through the blood stream. In contrast, agents directed at the tumor cells often do not adequately penetrate into the tumor. Second, tumor cells depend on blood supply; an average of 100 tumor cells depends on one endothelial cell, making vascular therapy potentially highly effective. Finally, the vasculature is composed of normal cells, which, because they do not possess the genetic instability characteristic of tumor cells, are unlikely to develop resistance to treatments.

Tumor vasculature grows as the tumor grows, and this process – angiogenesis – makes tumor blood vessels distinct from normal resting blood vessels. Several anti-angiogenic therapies are in pre-clinical and clinical development. The approach we are working on, while it also targets the blood vessels, differs from these anti-angiogenic therapies in many important ways. The therapeutic agent is concentrated in tumor vessels, but it acts both on the endothelial cells and the tumor cells. Furthermore, the therapy can be directed specifically to breast cancer vasculature – both the blood vessels and the lymphatic vessels – eliminating potential side effects from targeting angiogenesis in tissue repair. Finally, we can also target pre-malignant lesions for destruction.

We identify tissue-specific and tumor-specific vascular markers by *in vivo* screening of libraries of peptides displayed on phage. The vasculature of normal breast tissue expresses specific markers that we can selectively target with the phage-derived peptides. Tumor vasculature can be targeted through its angiogenesis-associated markers or blood vessel and through lymphatic markers specific for individual tumor types. In this project, we profile the specialization of the vasculature, both blood vessels and lymphatic, in pre-malignant and malignant breast lesions as well as metastases. The tools developed in this work can be used to design new therapies that specifically target such lesions.

## BODY

The approved tasks for this project are:

**Task 1:** To identify peptides that specifically home to the blood and/or lymphatic vessels of MMTV-PyMT breast cancers.

**Task 2:** To validate the specific blood vessel and lymphatic vessel homing of the selected peptides.

**Task 3:** To identify and characterize receptors for the peptides that specifically home to the blood and/or lymphatic vessels of breast cancers.

**Task 4:** Study the specificity of vascular entry of tumor cells by using phage libraries.

**Task 5:** To study the use of homing peptides in breast cancer prevention and treatment in mouse models.

During the first year of grant support we have made substantial progress with each of the Tasks:

*Tasks 1 and 2.* We have established the MMTV-PyMT breast cancer model in our laboratory and have performed the first round of screening with these tumors. The most interesting peptide that emerged from this screening is a pentapeptide with the sequence CREKA. The CREKA phage and fluorescein-labeled CREKA peptide home to tumors, where our results suggest they bind to type IV collagen in tumor blood vessels and tumor matrix (Fig. 1). Others have shown that the mRNAs for several collagen chains, including type IV, are greatly (more than 30-fold) elevated in tumor endothelial cells compared to endothelial cells from adjacent normal tissue (St. Croix *et al. Science 289*, 1197-1202, 2000). This quantitative difference alone may explain the selective homing of our phage and labeled peptide to tumors (Fig. 2). It is also possible that tumor collagens are poorly assembled in tumor tissue and expose more binding sites for the peptide. Further characterization of the CREKA peptide is underway, as is further screening of the PyMT tumors for additional peptides.

**A**

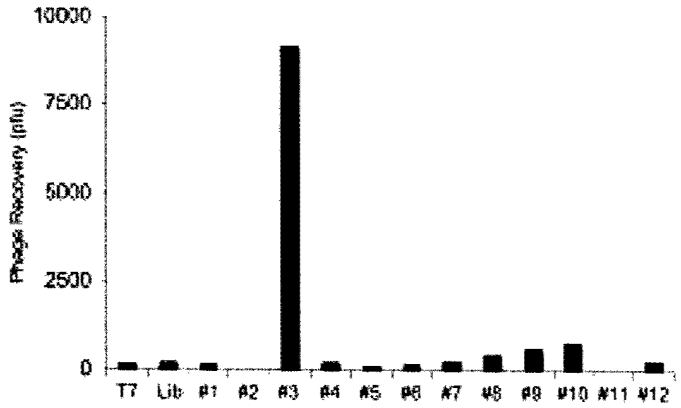


**Fig. 1. Specific binding of CREKA phage to a type IV collagen fragment and homing of fluorescein-labeled CREKA peptide to MMTV-PyMT breast cancer. cDNA clone encoding a CREKA-binding protein fragment. (A)** Biotin-labeled CREKA was immobilized on Streptavidin-coated ELISA plates. A cDNA library from mouse breast carcinoma in T7 phage ($1 \times 10^8$ pfu) was applied to the CREKA-coated surface. The wells were washed with PBS, and specifically bound phage was eluted with an excess of soluble CREKA peptide. The eluted clones were amplified and individually tested with non-recombinant T7 phage (T7) and the original cDNA library (Lib) for binding to CREKA coated wells. Clone #3 phage avidly bound to CREKA. **(B)** The cDNA from the CREKA-binding phage encodes a 138-amino acid fragment with a sequence identical to the collagen IV alpha 2chain. The numbers that bracket the alignment denote the amino acid residues in collagen IV alpha 2 chain (NCBI accession number P08122) that align with clone #3.

**B**

| | | |
|---|---|---|
| **Clone #3** | | GERGEQGPPGPSVYSPHPSLAKGARGDPGFQGAHGEPGSRGEPGEPGTAG |
| **Collagen alpha 2 (IV)** | 336 | **GERGEQGPPGPSVYSPHPSLAKGARGDPGFQGAHGEPGSRGEPGEPGTAG** 385 |
| **Clone #3** | | PPGPSVGDEDSMRGLPGEMGPKGFSGEPGSPARYLGPPGADGRPGPQGVP |
| **Collagen alpha 2 (IV)** | 386 | **PPGPSVGDEDSMRGLPGEMGPKGFSGEPGSPARYLGPPGADGRPGPQGVP** 435 |
| **Clone #3** | | GPAGPPGPDGFLFGLKGSEGRVGYPGPSGFPGTRGQ - AW |
| **Collagen alpha 2 (IV)** | 436 | **GPAGPPGPDGFLFGLKGSEGRVGYPGPSGFPGTRGQKGW** 474 |

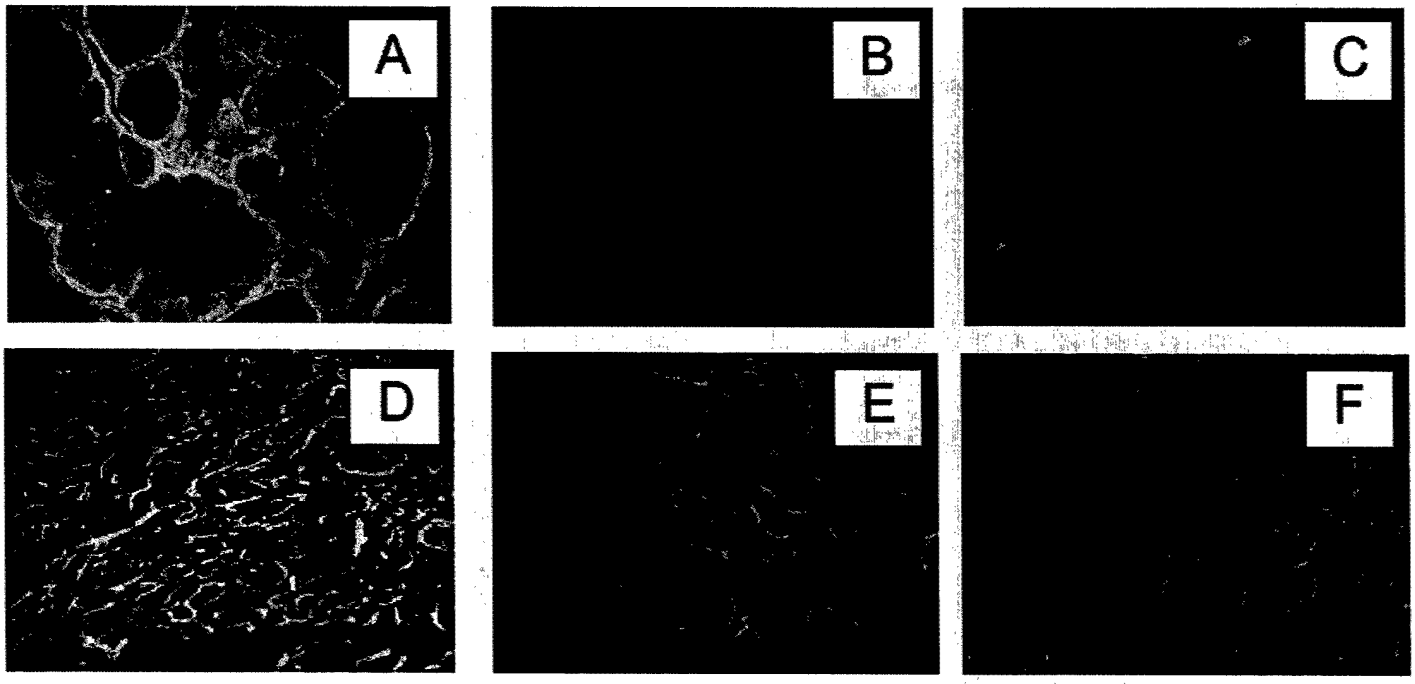


**Fig. 2. Fluorescein-labeled CREKA peptide homes to tumors, but not to normal tissues.** A-F, Localization of FITC-labeled and rhodamine-labeled CREKA peptide in MMTV -PyMT tumors. Fluorescein-labeled CREKA peptide (FITC-CREKA; 100 mg in 100 ml PBS) was injected into the tail vein of MMTV PyMT mice. After 15 min (A-C) or 2 hrs (D) of circulation, the mice were perfused through the heart. Organs were dissected, fixed, and cryo-sections were examined by fluorescence microscopy. (A) FITC-CREKA fluorescence was present in MMTV PyMT tumors 15 min after the injection. The peptide was primarily located in the periphery of the tumor. No peptide was detected in control organs such as the brain (B), or liver (C). After 2 hrs of circulation, rhodamine-CREKA was present throughout the tumor, localizing outside of the vasculature (D). In (D), nuclei were counter-stained with DAPI and tumor vasculature was visualized with intravenous injection of FITC-tomato lectin. Tumor vasculature visualized with intravenous injection of FITC-tomato-lectin (E). Heart from a MMTV PyMT mouse injected with rhodamine-CREKA and FITC tomato-lectin counter-stained with DAPI (F).

We had previously shown that the lymphatic vessels in a human breast cancer xenograft carry a specific marker detectable with a peptide isolated from a phage library (Laakkonen *et al.*, 2002). The peptide, LyP-1, homes to lymphatic vessels in the breast cancer xenografts and in some other tumors. However, some other tumors (particularly xenografts obtained with the human melanoma C8161 cells), even though they contained lymphatic vessels, were negative. We screened phage libraries for peptides that would bind to the lymphatics in the C8161 tumors. We then developed a new screening procedure for this purpose that is based on isolation of lymphatic endothelial cells (and phage bound to them) with antibodies that specifically recognize these cells. We have identified two lymphatic homing peptides for this tumor, but they home poorly, or not at all to the MDA-MB-435 tumors. Thus, a lymphatic vessel "zip code" system of the kind predicted in the original application is beginning to emerge.

We will next use the PyMT transgenic tumor model for the lymphatic screening for two reasons. First, this tumor model may more closely resemble human breast cancer than the xenografts. Second, these tumors grow in the mammary fat pad, and the normal lymphatics in this tissue may be different from those in the skin, where the xenografts are grown. The TRAMP transgenic prostate cancer model will provide a control for internal (non-skin) tumor lymphatics in these experiments.

*Task 3.* We have identified cell surface-expressed nucleolin as a receptor for a tumor-homing peptide on the MDA-MB-435 cells. The peptide, F3, is a fragment of the nuclear protein HMGN2 (Porkka et al., 2002). F3 binds to tumor blood vessel endothelial cells and tumor cells and it also recognizes a small subpopulation of cells in the bone marrow that may represent endothelial precursor cells. These results, which are being prepared for publication, establish nucleolin as a marker of tumor vessels. As F3 is taken up by the cells to which it binds and is transported into their nucleus, the results also suggest that nucleolin may serve as a shuttle between the cell surface and the nucleus.

*Task 4.* Our initial studies on the use of phage libraries to study tumor intravasation were hampered by varying uptake of the phage by the liver and spleen (presumably by the reticuloendothelial system). We were not able to reliably distinguish phage that were preferentially transported from tissue into the blood from phage that was not efficiently eliminated by the reticuloendothelial system. We have now selected phage that is not eliminated into the reticuloendothelial system, or does it slowly. We have this phage to construct peptide libraries and will be using these libraries to screen for enhanced intravasation as described in the application.

*Task 5.* In preparation for treatment experiments, we have shown that the LyP-1 peptide, which homes to tumor lymphatics, accumulates in MDA-MB-435 xenografts with an extraordinary efficiency and selectivity. In fact, we are able to detect the fluorescein-labeled LyP-1 in tumors of intact mice after an intravenous injection (Fig. 3). The fluorescence in the subcutaneous tumors is detectable under UV light through the skin, whereas other tissues are completely negative in this examination and in tissue sections. These results will help us achieve maximal concentration or drugs in tumors in future experiments.

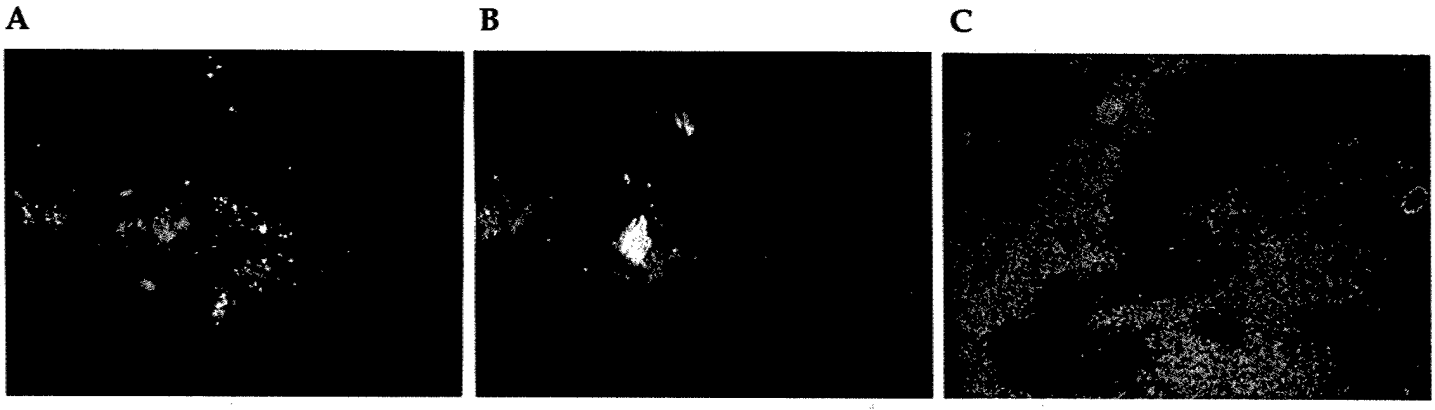


**Fig. 3. Accumulation of fluorescein-labeled LyP-1 peptide in breast cancer xenografts.** Mice bearing MDA-MB-435 cell xenografts were intravenously injected with fluorescein-labeled LyP-1 peptide (A) or similarly labeled control peptide (B). The tumors were viewed under UV light in the intact mice. The tumor of the LyP-1 injected mouse shows bright specific fluorescence. (C) A section from the tumor of a mouse injected with fluorescence-labeled LyP-1 (as in panel A).

## KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that Quantum dots (semiconductor material nanocrystals) coated with homing peptides specifically accumulate in breast cancer xenografts *in vivo*.
- Identified a peptide that binds to type IV collagen in breast cancer vasculature and in tumor extracellular matrix by in vivo phage screening.
- Isolated additional peptides that home to lymphatic vessels in tumors (including breast cancer xenografts).
- Identified a breast cancer cell receptor for a peptide that homes to tumor vasculature.
- Identified a T7 phage mutant that is not eliminated into the liver and spleen.
- Shown that a fluorescein-labeled peptide that recognizes lymphatic vessels in breast cancer xenografts specifically accumulates in these tumors with an unprecedented efficacy after an intravenous injection. The accumulation is sufficient to allow the tumor fluorescence to be detected non-invasively.

## REPORTABLE OUTCOMES

One original paper and one review acknowledging this grant have been published:

Akerman, M.E., Chan, W.C.W., Laakkonen, P., Bhatia, S.N., and Ruoslahti, E. Nanocrystal targeting in vivo. *Proc. Natl. Acad. Sci. USA.* 99: 12617-12621 (2002). This paper was widely noted in the scientific and lay press (e.g. *Nature Materials*, September 19, 2002, electronic issue)

Ruoslahti, E. Drug targeting to specific vascular sites. *Drug Discovery Today.* **7**:1138- 1143 (2002).

## CONCLUSIONS

We have made significant progress toward completing each of the Tasks in the application. A "zip code" system for lymphatic vessels in tumors is beginning to emerge. This heterogeneity of the lymphatics is similar to what we have established and continue to establish for tumor blood vessels. One of the lymphatic homing peptides and a new blood vessel homing peptide both show remarkably efficient and specific accumulation in breast cancer xenografts, boding well for future drug targeting experiments.

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Laakkonen, P., Porkka, K., Hoffman, J. A., and Ruoslahti, E. A tumor-homing peptide with a lymphatic vessel-related targeting specificity. *Nature Med* 8: 743-751 (2002).

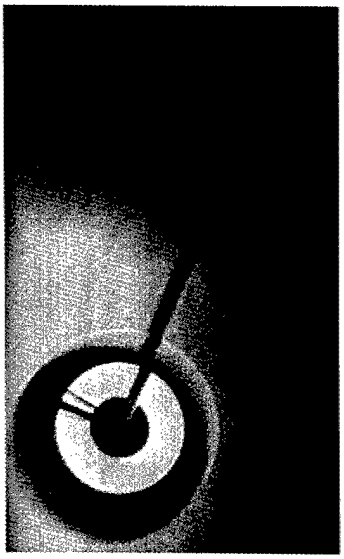
St Croix, B., Rago, C., Velculescu, V., Traverso, G., Romans, K. E., Montgomery, E., Lal, A., Riggins, G. J., Lengauer, C., Vogelstein, B. & Kinzler, K. W *et al.* Genes expressed in human tumor endothelium. *Science* 289: 1197-202 (2000).

## Nanoparticles hit the target

**Nanoparticles of metals and semiconductors have potential uses in biomedical diagnostics and therapies. By coating them with certain peptides they can be targeted to specific tissue types in the body.**

*19 September 2002*

**Philip Ball**



Nanoparticles can be delivered to specific biological tissues *in vivo* by giving them appropriate molecular 'hooks', researchers in the USA report. They believe that this type of tissue targeting should be useful for medical imaging applications, and also for drug delivery and other forms of therapy.

Inorganic nanoparticles made from metals and semiconductors are already used for biological imaging. Molecules tagged with gold nanoparticles, for example, show up in electron microscopy, and fluorescent emission from particles of II-VI or III-V semiconductors (such as zinc or cadmium sulphide) can be exploited in fluorescence optical microscopy. This fluorescent emission is bright, does not suffer from the photobleaching that can afflict molecular tags such as green fluorescent protein, and can be tuned between the infrared and the ultraviolet by altering the size of the nanoparticles. This tuning is a quantum effect, hence these particles are known as quantum dots.

It is often necessary for this diagnostic labelling with soluble nanoparticles to be confined to a particular tissue type. In particular, making quantum dots that will stick only to tumour cells would be very useful for imaging cancers. Erkki Ruoslahti of the Burnham Institute in La Jolla, California, and colleagues have found a way to give nanoparticles this kind of tissue selectivity[1].

They attach hooks to the nanoparticles that latch onto the vascular system of particular tissues or organs. The hooks are 'homing peptides' that bind to molecular markers expressed on the blood vessels. For example, one such peptide (denoted GFE) recognizes a membrane enzyme on the endothelial cells of lung blood vessels. Another, called F3, binds specifically to blood vessels and other cells in tumours. A third, called LyP-1, recognizes lymph vessels in certain tumours.

Ruoslahti and colleagues set out to establish whether quantum dots coated with these peptides could be targeted to the respective tissues, without indiscriminately binding to other cell types. They used nanoparticles of cadmium selenide coated with zinc sulphide and capped with water-soluble organic groups. The nanoparticles were monodisperse and less than 10 nm across, the precise size being varied to alter the emission wavelengths between red and green.

To attach the peptides, the researchers first coated the quantum dots with mercaptoacetic acid. These molecules could be exchanged for thiolated peptides when mixed together at room temperature, producing nanoparticles covered with the dangling peptide recognition groups.

When injected into the tails of mice, the nanoparticles found their way to their respective targets. Particles with grafted GFE peptides accumulated in the lungs, and were not found in various other tissues such as the brain and kidneys. Those particles with F3 and LyP-1 appendages became localized in human breast carcinoma tumours xenografted into the mice.

All the quantum dots, however, were also found in the liver and spleen. This shows that some of them were being spotted by the biological patrol system, the phagocyte cells of the reticuloendothelial system that acts as a filter for foreign particles. These cells are non-specific in their interaction with such particles, and transport them to the liver and spleen for clearance from the body.

But this patrol can be evaded. Ruoslahti and colleagues knew that coatings of polyethylene glycol (PEG) can minimize recognition by the reticuloendothelial system, and so they prepared quantum dots with a mixture of a homing peptide and PEG grafted on the surface. They found that attaching some PEG to LyP-1 quantum dots reduced the accumulation in the liver and spleen by 95%.

The researchers envisage making other kinds of targeted nanoparticles this way, such as magnetic particles for non-invasive magnetic probes. Particles containing encapsulated drugs, such as dendrimers or perhaps even nanocrystals of the drugs themselves, could be used to deliver the drugs selectively to the correct tissues. They even imagine more complex nanoscale assemblies that are capable of a 'smart' response, sensing the presence of disease or malfunction in a specific cell type and releasing the necessary drug just at that site.

**References**

1. Akerman M. E. , Chan W. C. W. , Laakkonen P. , Bhatia S. N. & Ruoslahti E. *Proceedings of the National Academy of Sciences* USA early edition online (2002) | article |

# Nanocrystal targeting *in vivo*

**Maria E. Åkerman*†‡, Warren C. W. Chan†‡, Pirjo Laakkonen*, Sangeeta N. Bhatia†, and Erkki Ruoslahti*§**

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Contributed by Erkki Ruoslahti, August 1, 2002

**Inorganic nanostructures that interface with biological systems have recently attracted widespread interest in biology and medicine. Nanoparticles are thought to have potential as novel intravascular probes for both diagnostic (e.g., imaging) and therapeutic purposes (e.g., drug delivery). Critical issues for successful nanoparticle delivery include the ability to target specific tissues and cell types and escape from the biological particulate filter known as the reticuloendothelial system. We set out to explore the feasibility of *in vivo* targeting by using semiconductor quantum dots (qdots). Qdots are small (<10 nm) inorganic nanocrystals that possess unique luminescent properties; their fluorescence emission is stable and tuned by varying the particle size or composition. We show that ZnS-capped CdSe qdots coated with a lung-targeting peptide accumulate in the lungs of mice after i.v. injection, whereas two other peptides specifically direct qdots to blood vessels or lymphatic vessels in tumors. We also show that adding polyethylene glycol to the qdot coating prevents nonselective accumulation of qdots in reticuloendothelial tissues. These results encourage the construction of more complex nanostructures with capabilities such as disease sensing and drug delivery.**

Hybrid organic/inorganic nanoparticles are thought to have potential as novel intravascular probes for diagnostics (e.g., imaging) and therapeutics (e.g., drug delivery) (1). For this potential to be realized, an ability to target the nanoparticles to specific tissues and cell types would be important. We used semiconductor quantum dots (qdots) coated with targeting peptides as prototypic nanostructures for intravascular delivery in live mice. Qdots are generally composed of atoms from groups II–VI or III–V of the periodic table and are defined as particles with physical dimensions smaller than the exciton Bohr radius (2). This size leads to a quantum confinement effect, which endows nanocrystals with unique optical and electronic properties. Qdots have size-tunable emission (from the UV to the IR), narrow spectral line widths, high luminescence, continuous absorption profiles, and stability against photobleaching (2–4). Furthermore, the large surface area-to-volume ratio of qdots makes them appealing for the design of more complex nanosystems.

Blood vessels express molecular markers that distinguish the vasculature of individual organs, tissues, and tumors. Peptides that recognize these vascular markers have been identified by screening phage libraries *in vivo*, a procedure in which peptides direct phage homing to individual sites (5). This approach has led to the identification of a unique set of homing peptides with high *in vivo* selectivity (6–9). We have used homing peptides to target i.v.-injected qdots to specific vascular sites in mice (Fig. 1). One of these peptides binds to membrane dipeptidase on the endothelial cells in lung blood vessels (9), and the other two preferentially bind to tumor blood vessels (10) or tumor lymphatic vessels (11) and the tumor cells. Each of the peptides directed the qdots to the appropriate site in the mice, showing that nanocrystals can be targeted *in vivo* with an exquisite specificity.

## Materials and Methods

**Preparation of Qdots and Peptide-Coated Qdots.** Previously published procedures were used to synthesize tri-*n*-octylphosphine oxide-coated ZnS-capped CdSe qdots (12–15) and to modify their surface chemistry to render them water soluble (16, 17). After this step, the surface of qdots was coated with mercaptoacetic acid.

Three peptides were used to coat qdots: CGFECVRQCPERC peptide (denoted as GFE) binds to membrane dipeptidase on the endothelial cells in lung blood vessels (9, 18), KDEPQRRSARLSAKPAPPKPEPKPKKAPAKK (F3) preferentially binds to blood vessels and tumor cells in various tumors (10), and CGNKRTRGC (LyP-1) recognizes lymphatic vessels and tumor cells in certain tumors (11). The peptides were synthesized by *N*-(9-fluorenylmethoxycarbonyl)-L-amino acids chemistry with a solid-phase synthesizer and purified by HPLC. The composition of the peptides was confirmed by MS.

The peptides were thiolated by using 3-mercaptopropionimidate hydrochloride (a.k.a. iminothiolane), an imidoester compound containing a sulfhydryl group. Peptides were incubated with iminothiolane for 1 h in 10 mM PBS, pH 7.4, at a 1:1 molar ratio. Afterward, mercaptoacetic acid-coated qdots were added to the solution to exchange some of the mercaptoacetic acid groups with the thiolated peptide incubated overnight at room temperature. For coadsorption of polyethylene glycol (PEG) and peptides, amine-terminated PEG (Shearwater Polymers, Huntsville, AL) was thiolated with iminothiolane. Thiolated PEG was directly added to a solution of mercaptoacetic acid-coated qdots in 10 mM PBS, pH 7.4, and allowed to incubate overnight at room temperature. Afterward, the thiolated peptide was added to the PEG/qdot solution and incubated overnight at room temperature. The coated qdots were purified with Microspin G-50 columns (Amersham Pharmacia) before assays or injection into a mouse. The coupling efficiency was determined by performing a Bradford assay (Bio-Rad) on the coated qdots.

**Mice, Cell Lines, and Tumors.** Lung endothelial (LE), brain endothelial, and human breast carcinoma MDA-MD-435 cells were maintained as described (7, 18). To establish tumor xenografts, $10^6$ exponentially growing MDA-MB-435 tumor cells were injected s.c. in the chest area of BALB/c *nu/nu* mice (Animal Technologies, Livermore, CA). The mice were used for *in vivo* targeting experiments 8–12 weeks after the tumor cell inoculation.

**Qdot Injections and Histology.** Peptide-coated qdots (100–200 $\mu$g in 0.1–0.2 ml PBS) were injected into the tail vein of a mouse and allowed to circulate for 5 min (GFE qdots) or 20 min (F3 and LyP-1 qdots). Blood vessels were visualized by i.v. injecting tomato lectin conjugated with either fluorescein or biotin (Vector Laboratories), as reported (11). While still under anesthesia, the mouse was perfused with 4% paraformaldehyde through the heart. Tissues were frozen in Tissue Tek OCT embedding medium (Sakura Finetek, Torrance, CA) before sectioning. The sections were mounted with Vectashield mounting medium with or without 4′,6-diamidino-2-phenylindole, dihydrochloride (Vector Laboratories) to visualize cell nuclei before examination under an inverted fluorescent microscope or a confocal microscope.



Abbreviations: qdot, quantum dot; PEG, polyethylene glycol; LE, lung endothelial.

‡M.E.Å. and W.C.W.C. contributed equally to this work.

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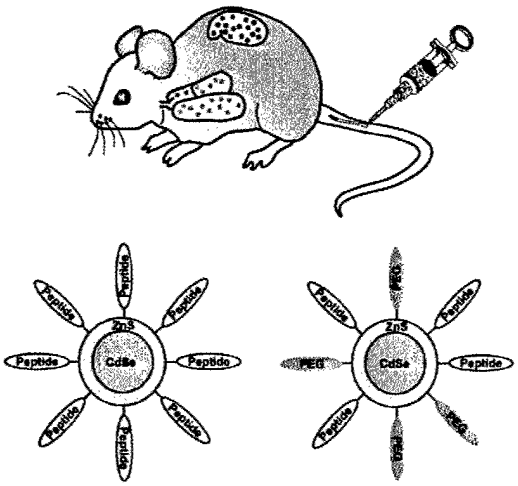


**Fig. 1.** Schematic representation of qdot targeting. Intravenous delivery of qdots into specific tissues of the mouse. (*Upper*) Design of peptide-coated qdots. (*Lower*) Qdots were coated with either peptides only or with peptides and PEG. PEG helps the qdots maintain solubility in aqueous solvents and minimize nonspecific binding.

## Results

**Peptide-Coated Qdots.** We synthesized ZnS-capped CdSe qdots emitting in the green and the red (550 nm and 625 nm fluorescence maxima, respectively) and coated them with peptides by using a thiol-exchange reaction. The coupling of the GFE peptides yielded monodisperse qdots, whereas qdots coated with the F3 aggregated. The large number of positive residues in F3 may have caused colloidal aggregation by "bridging" negatively charged qdots. We overcame the aggregation problem by either decreasing the population of peptides on the qdot surface or by co-coupling peptides and PEG (molecular weight = 5,000 g/mol), a polymer known to minimize molecular interactions and improve colloidal solubilities (19) (Fig. 1). Protein assays revealed about 120 peptide molecules per qdot when peptide only was coupled. Co-coupling of PEG reduced this number to about 70.

**Peptide Specificity *in Vitro*.** To explore the binding activity and specificity of peptide-coated qdots, experiments were first conducted *in vitro*. LE and brain endothelial cells were grown in culture, and green-luminescent GFE-coated qdots were incubated with each cell type. The LE cells express membrane dipeptidase, the receptor for the GFE peptide, whereas the brain endothelial cells do not (9). On optical excitation, qdots coated with the lung-homing GFE peptide were observed decorating the surface of LE cells (Fig. 2*a*), whereas no visible signal was observed on the brain endothelial cells. Specificity of the GFE-qdot binding to LE cells was further demonstrated by inhibition of binding by both the addition of free GFE peptide or the organic molecule cilastatin, a known ligand and inhibitor of membrane dipeptidase (20) (Fig. 2 *b* and *c*). This finding was quantified by using digital image analysis (Fig. 2*d*).

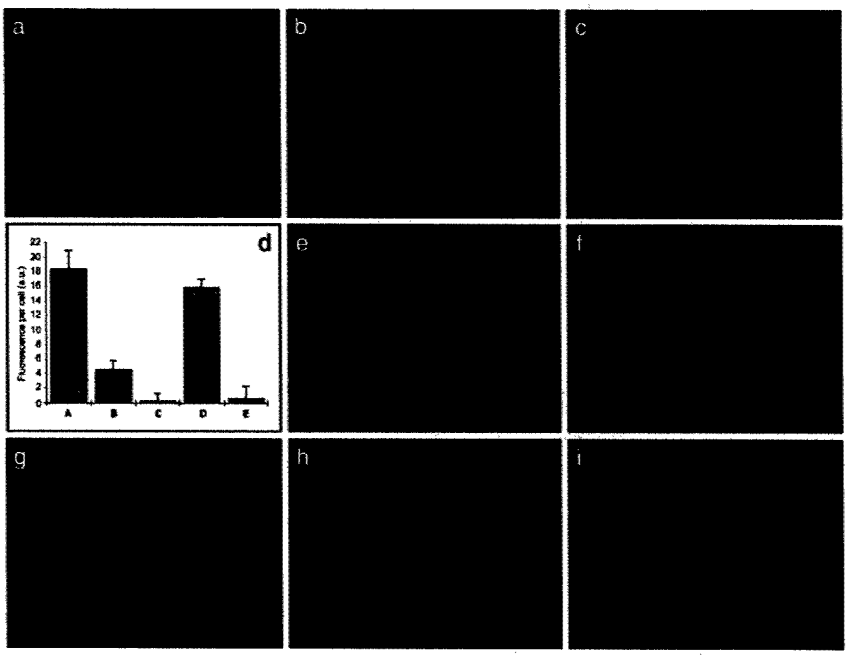


**Fig. 2.** Binding of peptide-conjugated qdots to endothelial cells and breast cancer cells *in vitro* is specific to peptide sequence. (*a*) Binding of green GFE-conjugated qdots to LE cells that express membrane dipeptidase. (*b* and *c*) Inhibition of GFE-qdot binding to LE cells by free GFE peptide (500 $\mu$M) (*b*) or with cilastatin, an inhibitor of the receptor, membrane dipeptidase (50 $\mu$M) (*c*). (*d*) Quantification of fluorescence intensity of an experiment similar to the one illustrated in *a*–*c*. (Columns A–C) GFE-qdot binding to LE cells; GFE concentration 250 $\mu$M; and cilastatin 50 $\mu$M. (Column D) The binding of GFE-qdots to the LE cells is not inhibited by a control peptide (LyP-1; 250 $\mu$M). (Column E) LyP-1-qdots do not bind to the LE cells. The fluorescence associated with 10 individual cells from each panel was measured by using digital image analysis. Background fluorescence from cells that received no qdots has been subtracted (2 a.u., arbitrary units). A representative experiment of five experiments was quantified. (*e* and *f*) F3 qdots bind to MDA-MB-435 breast carcinoma cells (*e*); free F3 peptide (500 $\mu$M) inhibits the binding (*f*). (*g*) Binding of LyP-1 qdots to MDA-MB-435 cells. (*h* and *i*) GFE qdots do not recognize the MDA-MB-435 cells (*h*) and LyP-1 qdots do not bind to the LE cells (*i*). Nuclei were visualized with 4′,6-diamidino-2-phenylindole staining (blue). Cells were examined under an epifluorescence microscope with a 425/40-nm excitation and a 515-nm long-pass filter. (Original magnifications: *a*–*c* and *i*, ×200; and *e*–*h*, ×400.)

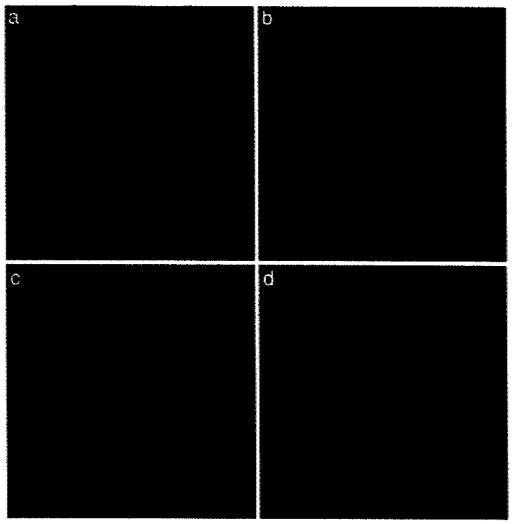


**Fig. 3.** *In vivo* targeting of qdots to normal lung vasculature is specific. Red GFE-conjugated qdots were injected into the tail vein of normal mice, and the presence of qdots in lung tissue was assessed by examining sections under a confocal microscope with UV excitation and a 585-nm long-pass filter (*a* and *b*) or an epifluorescent microscope as in Fig. 2 (*c* and *d*). (*a*) Red qdots localized in lung tissue. (*b*) Inhibition of qdot accumulation in the lungs by coinjected cilastatin. (*c* and *d*) Absence of GFE qdots in the brain (*c*) and kidney (*d*) demonstrates the specificity of binding. The results shown are representative of three experiments carried out with nine mice. (Original magnifications: *a* and *b*, ×600; *c* and *d*, ×200.)

Qdots coated with F3 or LyP-1 bound to the MDA-MB-435 human breast carcinoma cells (Fig. 2 *e* and *g*). As with GFE, specificity of F3- and LyP-1-coated qdots was demonstrated by inhibition of binding by the appropriate cognate peptide (shown for the F3 qdots in Fig. 2*f*), and further confirmed by lack of inhibition by an unrelated (GFE) peptide or by cilastatin (not shown). The GFE-coated qdots did not bind to the MDA-MB-435 cells (Fig. 2*h*), and LyP-1-coated qdots did not bind to the endothelial cells (Fig. 2*i*). These experiments show that coating with GFE, F3, or LyP-1 peptides endows qdots with specific affinity to their corresponding cellular target *in vitro*.

**Qdot Homing to Lung Endothelium *in Vivo*.** Next, the ability of peptide-coated qdots to home to their targets *in vivo* was examined. Because red luminescent qdots were easier to distinguish from the autofluorescent tissue background than the green luminescent qdots, we i.v. injected red qdots coated with GFE into normal BALB/c mice and studied the tissue distribution of the injected qdots 5 min later. We detected bright GFE-qdot luminescence in the lungs (Fig. 3*a*), and its appearance was inhibited when the qdots were coinjected with cilastatin, similar to the *in vitro* studies (Fig. 3*b*). The GFE qdots were not found in various other organs (Fig. 3*c*, brain and Fig. 3*d*, kidney), except those with a prominent reticuloendothelial component (see below). Furthermore, we did not observe any acute toxicity, even after 24 h of circulation, caused by the i.v. administration of these nanoparticles (i.e., overt thrombosis or signs of complement activation).

**Qdot Homing to Tumor Vasculature *in Vivo*.** Qdots coated with peptides that home to tumor vasculature were examined in an MDA-MB-435 xenograft tumor system. Intravenously injected F3-coated and LyP-1-coated qdots accumulated in these tumors. As expected from their specificity for tumor blood vessels (10) and lymphatic vessels (11), the F3 and LyP-1 qdots targeted distinct structures within the tumors. The signal from F3-coated qdots colocalized with coinjected blood vessel marker lectin (Fig. 4*a*). The LyP-1-coated qdots did not

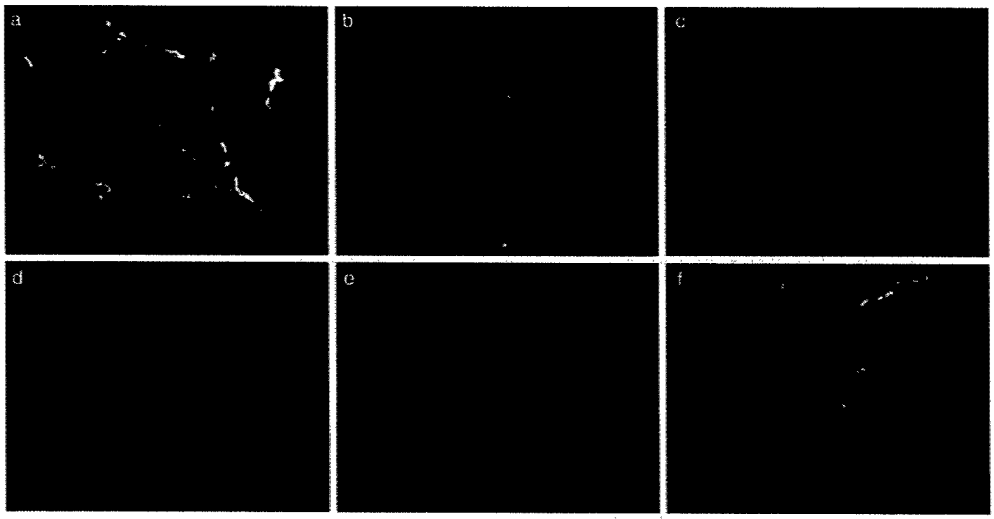


**Fig. 4.** *In vivo* targeting of qdots to tumor vasculature is specific. Red F3 or LyP-1 qdots, both PEG-coated, were injected into the tail vein of nude BALB/c mice bearing MDA-MB-435 breast carcinoma xenograft tumors. Blood vessels were visualized by coinjecting tomato lectin (green). (*a*) F3 qdots colocalize with blood vessels in tumor tissue. (*b*) LyP-1 qdots also accumulate in tumor tissue, but do not colocalize with the blood vessel marker. (*c*) Red F3 qdots and green LyP-1 qdots injected into the same tumor mouse target different structures in tumor tissue. (*d*) GFE qdots that bind to normal LE injected into tumor mice are not detected in tumor tissue. (*e*) F3 qdots injected into tumor mice do not appear in the skin taken from an area next to the tumor. A longer exposure was used in *d* and *e* to bring out the tissue background. (*f*) LyP-1 qdots are internalized by cells in tumor tissue. Images *a*, *b*, and *f* were obtained with a confocal microscope, and images *c*–*e* were obtained with an epifluorescent microscope as in Fig. 2. The results shown are representative of experiments carried out with six mice for the F3 qdots and 12 mice for the LyP-1 qdots. (Original magnifications: *a* and *d*, ×400; *b* and *c*, ×600; *e*, ×200; and *f*, ×2,400.)

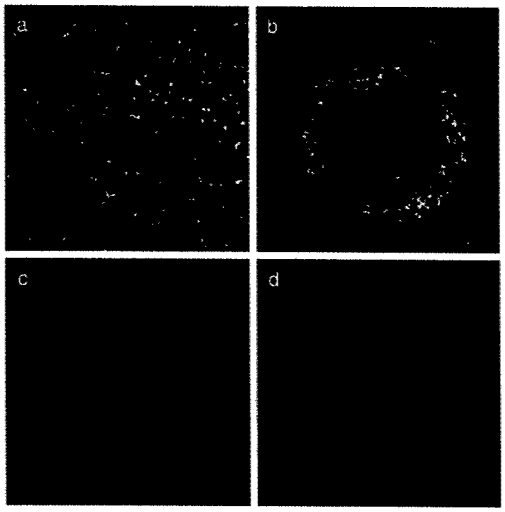


**Fig. 5.** Qdot uptake by the reticuloendothelial system is reduced by PEG coating. Green LyP-1 qdots with or without an added PEG coating were adjusted to an equal concentration by measuring absorbance at 540 nm and injected into the tail vein of mice bearing MDA-MB-435 tumors. Qdot localization in reticuloendothelial tissues was studied by epifluorescent microscopy of tissue sections as in Fig. 2. (*a* and *b*) LyP-1 qdots in the liver (*a*) and spleen (*b*). (*c* and *d*) LyP-1/PEG qdots in the liver (*c*) and spleen (*d*). These images are representative of three independent experiments. (Original magnifications: ×200.)

colocalize with the lectin (Fig. 4*b*) or F3 qdots (Fig. 4*c*). They did colocalize with the lymphatic vessel marker, podoplanin (21) (not shown), as has been shown previously for fluorescein-labeled LyP-1 peptide (11). GFE-coated qdots injected as a control were not detected in the tumors (Fig. 4*d*). Brain, heart, kidney, or skin did not contain detectable qdots (Fig. 4*e* and data not shown), indicating specificity of the F3 and LyP-1 qdots for tumor components. The regional specificity of qdot delivery within a tumor demonstrates the feasibility of targeting functionally distinct components of a tumor (e.g., blood vessels vs. lymphatics, etc.). Furthermore, the subcellular pattern of qdot luminescence suggests that peptide-coated nanostructures are internalized after binding to the cell surface, which may have implications for drug delivery and other applications that require intracellular targeting (Fig. 4*f*).

**Elimination of the Nonspecific Uptake by the Reticuloendothelial System.** The *in vivo*-injected qdots, regardless of the peptide used for the coating, accumulated in both the liver and spleen, in addition to the targeted tissues. The mononuclear phagocytes of the reticuloendothelial system, characterized by their ability to mediate nonspecific uptake of circulating particulates, apparently participated in the clearance of a fraction of circulating qdots in our experimental system. Adsorption-resistant coatings such as PEG are often used to minimize recognition by the reticuloendothelial system, thereby increasing circulation half-life (22). We prepared LyP-1 qdots with and without PEG coating and found that PEG nearly eliminated the nonspecific uptake into the liver and spleen (Fig. 5). Based on quantification of qdot fluorescence with digital image analysis, we estimate that coadsorption of PEG with peptide on the surface of qdots reduced the accumulation in the liver and spleen by about 95%. The PEG coating did not noticeably alter qdot accumulation in tumor tissue.

## Discussion

In this article, we demonstrate the selective targeting of peptide-coated qdots to the vasculature of normal lungs and tumors, showing that it is possible to target hybrid organic/inorganic nanometer-sized colloidal material in a living mammal. In the future, the components and functions of a nanosystem will not be limited to peptide/semiconductor composites and luminescence.

Our peptide-coated qdots showed excellent homing specificity for the relevant vascular site, but we did not see accumulation of fluorescence within the targeted tissue. This finding is in contrast to what we have seen when the two tumor-homing peptides, F3 and LyP-1, were coupled to fluorescein. In that case, there was accumulation of fluorescence not only in the blood or lymphatic vessels, but also in the tumor cells. It is also possible that qdots did not penetrate into the tissue. The uncoated qdots have a diameter of 3.5 nm (green) or 5.5 nm (red) (14), which is equivalent to about 40 kDa, and the peptide coating adds another about 150 kDa. This may be a sufficiently large size to impede tissue penetration. However, F3 and LyP-1 qdots accumulated less well in cultured cells than the fluorescein-labeled peptide. It may be that the qdots were not sufficiently stable to remain luminescent in living cells and tissues. Alternatively, the fluorescence may be quenched by the qdots subjected to low pH in the microenvironment, by oxidation of the surface, or by factors adsorbed to the surface. For the delivery of nanocrystalline drugs, crystals that are unstable in tissue may offer an advantage, as the drug would dissolve at the target.

Our results suggest the potential selective targeting of other nanomaterials [e.g., optically active metallic colloids (23), near-IR emitting nanocrystals (24–26), and magnetic nanoparticles (27, 28)] as *in vivo* optical and magnetic probes for noninvasive imaging. The use of peptides to target drug-carrying nanostructures [such as those composed of fullerenes (29, 30) or dendrimers (31, 32), or stabilized drug nanocrystals] should also be possible. This targeting approach has been used recently to deliver nano-sized particles composed of lipids and DNA to tumor vasculature (33). Although the current nanosystems are rather simple, in the future we envision the fabrication of multifunctional nanosystems, known as nanomachines. Such devices may, as an example, sense the presence of disease, deliver a drug to the site of disease, and release the drug at that site.

We thank Drs. Darren Brown, Fernando Ferrer, Edward Monosov, Lea Rudee, and Michael Sailor for their help with this work. Oriaku A. Kas-Osoka assisted with qdot synthesis, and Robbin Newlin assisted with histology. This study was supported by grants from the National Cancer Institute (CA82713), the Department of Defense (DAMD 17-02-1-0315), and the Komen Foundation (99–3339) (to E.R.), and the David and Lucile Packard Foundation and the Defense Advanced Research Projects Agency (to S.N.B.). M.E.A. received support from the American-Scandinavian Foundation; W.C.W.C., received support from the National Institutes of Health; and P.L., received support from the Academy of Finland and the Finnish Cultural Foundation.

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# Drug targeting to specific vascular sites

Erkki Ruoslahti

The blood vessels of individual tissues are biochemically distinct, and pathological lesions put their own signature on the vasculature. In tumors, both blood and lymphatic vessels differ from normal vessels. New methods, such as *in vivo* screening of phage libraries, have provided peptides and antibodies that recognize these vascular signatures and can be used in targeted delivery of therapeutic agents. Targeting a therapy to the diseased tissue enhances the efficacy of the treatment while reducing the side effects in mouse experiments. Results from drug delivery to tumor vessels have been particularly encouraging.

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▼ The vasculature of individual tissues expresses molecules that are specific to a particular tissue or tissue type. A well-known example is the blood vessels of lymphoid tissues, in which the high endothelium expresses unique adhesion molecules for lymphocyte homing. Many, perhaps all, other tissues are now also known to put a 'signature' on their vasculature [1].

Pathological tissue processes can also put their own signature on the vasculature. Inflammation and malignancy are known to do this. Tumor vasculature continuously undergoes angiogenesis to provide the blood supply that feeds the growing tumor [2]. The activated endothelial cells and pericytes in this neovasculature express molecules that are characteristic of angiogenic vessels (not expressed or expressed at much lower levels in normal vessels). Moreover, tumor lymphatics can also express their own marker. The heterogeneity in the vasculature might provide new opportunities for targeted delivery of therapies. This review discusses these developments.

## Molecular markers in blood vessels and lymphatics

*Blood vessel specialization in normal tissues.* Recently, an unexpected complexity of tissue-specific molecular individuality in vascular beds was uncovered. Monoclonal antibodies (MAbs) and a new technique using *in vivo* screening of peptide and antibody libraries expressed on the surface of phage or bacteria, have been particularly informative in this regard (reviewed in Ref. [1]). Intravenous injection of mice with peptide libraries displayed on phage, followed by isolation of phage from individual tissues has yielded tissue-specific vascular homing peptides for each normal tissue and organ our laboratory has chosen for targeting so far. Preparation of MAbs against specific membrane fractions from endothelia has yielded additional tissue-specific blood vessel markers [3,4].

*Special features of blood vessels in pathological tissues.* Tumor blood vessels express specific markers that are not present in the blood vessels of normal tissues. Such marker molecules can be present in the endothelial cells, the pericytes or the extracellular matrix (ECM) of tumor blood vessels. Many of the marker molecules that are selectively expressed in tumor blood vessels are proteins associated with tumor-induced angiogenesis, the sprouting of new blood vessels [5]. These proteins are often functionally important in the angiogenesis process; agents that perturb their function suppress angiogenesis. Tumor blood vessels are prime targets for inhibiting tumor growth. Because these vessels are distinct from normal resting blood vessels, they can be selectively destroyed without significantly affecting normal vessels. Inflammatory lesions such as arthritic synovium are also angiogenic and can be targeted with phage-displayed peptides [6]. Atherosclerotic plaques is another lesion in which specific molecular markers are detectable with *in vivo* peptide library screening [7].

*Endothelial marker molecules.* The molecular nature of vascular changes that give rise to the individuality of the vessels in various tissues and pathological lesions is partially understood. Several proteases have been identified as markers of the vasculature in individual normal tissues (Table 1). Thus, two peptidases


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(dipeptidyl peptidase IV [8] and membrane dipeptidase [1]) and a chloride channel are selectively expressed in lung vessels [4]. Another peptidase, aminopeptidase P, is a marker for breast gland vasculature [9].

The molecular markers of angiogenesis include additional peptidases or proteases and integrins. Aminopeptidase N is a marker of angiogenic vessels. It is a membrane-spanning 140-KDa cell surface protein that has previously been linked to cell migration and tumor invasion, but is a new marker of angiogenic endothelium. Antibodies against aminopeptidase N and enzymatic inhibitors of this enzyme block angiogenesis [10].

The cell adhesion receptors, integrins αvβ3, αvβ5 and α5β1 are over-expressed in tumor vasculature [11,12]. Indeed, one of the peptides identified by *in vivo* screening of phage libraries for tumor homing recognizes αvβ3 and αvβ5 [Table 1; 13]. Antibodies and peptides specific for these integrins have been used as receptors for targeted delivery of anti-cancer and anti-angiogenic agents (see below).

Serial analysis of gene expression has been used to survey differences in mRNA expression between endothelial cells from human colon cancers and from adjacent normal tissue [14]. Certain ECM proteins, particularly collagens, were found to be expressed at 10- to 30-fold higher levels in tumor endothelial cells than those from the normal tissue. Some matrix metalloprotease mRNAs were also over-expressed in tumor vasculature. Yet another set of differences included 'new' tumor-specific molecules, related only to expressed sequence tags in the databases. Some of these tumor endothelial markers (TEMs) had apparent transmembrane domains, which suggests that the proteins are expressed on the cell surface. One of the TEMs is endosialin, independently shown by another group to be a marker of angiogenic blood vessels [15].

Genetic programs initiated by angiogenic growth factors produced by tumor tissue (and other tissues that require neovascularization) are likely to be responsible for the production of the angiogenesis-related markers in tumor vasculature. Upregulation of tissue factor (TF) expression in tumor endothelial cells is also likely to occur under the influence of the tumor tissue, as TF is not expressed on endothelial cells of normal vessels [16]. TF binds factor VII to initiate blood clotting, and its expression by tumor endothelia is thought to contribute to the increased incidence of thrombosis seen in cancer patients. Further analyses of tumor vessels are likely to uncover markers that are specific for individual tumor types.

*Markers in pericytes and ECM.* Molecular markers of angiogenesis are not limited to the endothelium. The supporting mural cells (pericytes and smooth muscle cells) and the ECM also carry distinct markers. NG2 proteoglycan, also known as melanoma-associated chondroitin sulfate proteoglycan, is one such marker. NG2 is a membrane-spanning cell surface protein that is expressed in the neovasculature of tumors, regenerating tissues and in fetal vessels [17]. NG2 expression is limited to pericytes; endothelial cells do not express detectable levels of NG2.

The ECM of blood vessels consists of a sub-endothelial basement membrane and the matrix surrounding the mural cells. The expression of one matrix component, an alternatively spliced form of fibronectin containing an additional type III domain, ED-B, is restricted to tumor vessels and vessels of non-malignant tissues undergoing angiogenesis [18]. Fibronectin promotes cell adhesion, migration, growth and survival; the specific function of its ED-B isoform is not known.

*Lymphatics in tumors.* The presence and importance of blood vessels in tumors is well-established, but it has only recently been found that lymphatic vessels can also be present within tumors. The abundance of lymphatic vessels in and around a tumor correlates with the propensity of that tumor to metastasize [19]. We have recently reported evidence indicating that lymphatic vessels in tumors can also be specialized. A nonapeptide, LyP-1, isolated by combining phage display *ex vivo* and *in vivo*, directs the homing of the phage to vessel-like structures in certain tumors [20]. These structures are not blood vessels because they stain negative for blood vessel markers. Instead, they stain for various markers of lymphatic vessels. LyP-1 recognizes these apparent lymphatic vessels in most, but not all, tumors. Fluorescein-labeled LyP-1 peptide also homes to tumors after an intravenous injection. The fluorescence appears in tumor lymphatics and accumulates in the nuclei of the lymphatic endothelial cells (Fig. 1). Fluorescent LyP-1 is not detected in normal tissues, indicating that this peptide distinguishes lymphatics in tumors from normal lymphatics of the same animal. In addition to the lymphatic endothelial cells, LyP-1 also binds to the tumor cells, accumulating in their nuclei. Thus, this peptide can selectively transport its payload (fluorescein) all the way from the systemic circulation to the nuclei of the target cells.

Tumor cells can cover a substantial fraction of the luminal surface of tumor blood vessels [21]. These tumor cells might be in the process of transmigrating through the blood vessel wall, rather than being a structural component of the vessel. Tumor cells might also form ECM-lined channels that have been proposed to function as an auxiliary vasculature in highly malignant tumors [22]. These findings suggest that peptides or antibodies that bind to tumor cells, rather than to the conventional vascular cells, might also be capable of targeting tumor vasculature.

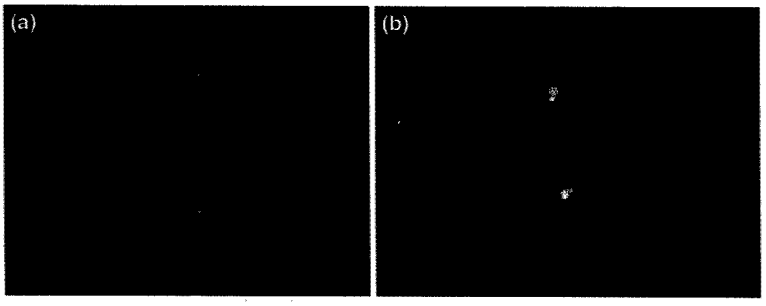


**Figure 1.** Targeting of tumor lymphatics with a fluorescein-labeled homing peptide. Intravenously injected LyP-1 peptide (sequence, CGNKRTRGC) homes to tumor tissue. **(a)** The peptide fluorescence (green) shows no overlap with blood vessels (red). **(b)** The peptide (green) appears in endothelial cells of a lymphatic vessel identified by podoplanin staining (red). The peptide accumulates in cell nuclei. Figure modified, with permission, from Ref. [20]. Magnification ×200.

*Origin of vascular specialization.* One can tentatively make two generalizations regarding tissue- and tumor-specific markers of vessels: these markers are likely to be functionally important to the tumor vessels and many of them represent special forms of otherwise common proteins. Integrins illustrate the first point. The αvβ3, αvβ5 and α5β1 integrins are each upregulated in angiogenic vessels and play a role in angiogenesis [11,12]. In the fetus, α5β1 is necessary for the development of the vasculature [23], whereas fetal or adult angiogenesis can take place without the αv integrins [24,25]. However, αvβ3 and αvβ5 are somehow important in adult angiogenesis because peptides and antibodies that perturb their function block angiogenesis [5,11]. Aminopeptidase N is another angiogenesis marker, which appears to be needed for angiogenesis to proceed [10].

The αvβ3 and αvβ5 integrins are essentially absent from the normal tissues of an adult animal and are selectively upregulated in angiogenesis. In contrast, aminopeptidase N is expressed in several epithelial tissues and in macrophages. Two factors might account for the homing specificity of the aminopeptidase N-binding peptides: (1) angiogenic vessels are the only vessels that express aminopeptidase N and (2) these vessels seem to express a specific form of this peptidase, as peptides that bind to aminopeptidase N in tumor vessels do not bind to macrophages [26]. In a similar vein, an antibody that recognizes a splice variant of fibronectin selectively recognizes the ECM of angiogenic blood vessels [18]. A peptide that homes to the vessels in normal breast tissue might similarly recognize a subset of aminopeptidase P molecules [9].

## *Delivery of therapeutic agents to vascular targets*

*Drug delivery.* Peptides, peptidomimetics and antibodies that home to a specific site in the vasculature are attractive as carriers of therapeutic and diagnostic agents. Specific drug delivery should concentrate the drug at the targeted site, increasing efficacy and also decreasing side effects in other tissues. Targeted delivery is particularly attractive in cancer therapy. The chemotherapeutic drugs currently used to treat cancer are highly toxic, which places a limit on the dose a patient can tolerate. Targeted delivery of high drug-concentrations to tumor tissue might alleviate this problem because normal tissue would be less affected. Moreover, targeting therapeutic agents to the vasculature of tumors, as opposed to the tumor cells themselves, might offer some additional advantages. Tumors are critically dependent on a blood supply; eliminating that supply can profoundly suppress tumor growth [2]. Blood vessels are more readily accessible to intravenously administered therapy than tumor cells. Furthermore, although tumor blood vessels acquire a tumor-associated 'signature', they are composed of normal cells that do not readily acquire mutations leading to drug resistance [27]. Moreover, when a targeted anti-angiogenic agent is also active against the tumor cells, additional gains in efficiency can be expected.

Proof for the vasculature-targeted delivery principle has been obtained in studies with experimental tumors. Researchers have designed compounds that can specifically deliver anti-tumor drugs to tumor vasculature. In one approach, investigators used vascular endothelial growth factor (VEGF) to target diphtheria toxin to VEGF receptors expressed by the tumor endothelial cells [28]. We have used homing peptides to construct drugs that bind to tumor vasculature. The RGD-4C peptide specifically binds to the αvβ3 and αvβ5 integrins [29], and the CNGRC peptide binds to aminopeptidase N [10]. Coupling of doxorubicin to the RGD-4C and CNGRC targeting peptides yielded compounds that were more effective and less toxic than doxorubicin alone [13]. Doxorubicin, like several other cytotoxic chemotherapeutics, inhibits angiogenesis in addition to being toxic to tumor cells [30]. The vascular targeting approach is likely to enhance these anti-angiogenic effects. The targeting of doxorubicin using the RGD-4C and CNGRC peptides reduced the side effects of the treatment in the heart and liver, which are the main sites of doxorubicin toxicity.

We have developed compounds that use a homing peptide for delivery and uptake into target cells, and a proapoptotic peptide as the drug component [31]. A major advantage of

**Table 1. Structure and activities of homing peptides identified by phage display[a].**

| | RGD-4C[b] | NGR[c] | LyP-1[d] | F3[e] | SMS[f] | GFE[g] |
|---|---|---|---|---|---|---|
| Sequence | CCDCRGDCFC | CCNGRC | cCGNKRTRGC | * | SMSIARL | cCGFECVRQCPERC |
| Cell surface | integrins αvβ3 and αvβ3 | amino- peptidase N | not known | not known | not known | membrane dipeptidase |
| Tissue | Angiogenesis | Angiogenesis | Tumor lymphatics/ tumor cells | Angiogenesis/ vessels | Prostate | Lung |
| Cellular localization of the peptide | Cell surface/ cytoplasmic | Cell surface/ cytoplasmic | Cell surface/ nuclear | Cell surface/ nuclear | Cell surface/ intracellular | Cell surface |

[a]The amino acid sequences of the peptides are given in the single letter code; c denotes cyclic structure formed by a disulfide bond.
[b]This is a cyclic nonapeptide containing two disulfide bonds; the form with the 1-3, 2-4 disuldide bond pattern is active in binding to the integrins [29].
[c]Peptides with the NGR motif bind to aminopeptidase N, which is specifically expressed in angiogenic vessels within the vasculature [10,13].
[d]A cyclic nonapeptide that recognizes tumor lymphatics and tumor cells in certain (but not all) tumors. This peptide is taken up by the cells it binds to and tranlocated into the nucleus [20].
[e]*A 31-amino acid peptide that binds to the endothelial cells in tumor blood vessels and tumor cells and is translocated into the nucleus [33].
[f]A linear heptapeptide that selectively binds to the vasculature of the normal prostate [32].
[g]A cyclic nonapeptide that recognizes membrane dipetidase, which is selectively expressed by lung blood vessels within the vasculature [39].

this class of compounds is that they can be prepared by solid phase peptide synthesis. In contrast, conjugation steps are required in joining a homing peptide with conventional drugs. The proapoptotic peptide is an anti-bacterial peptide that disrupts the membranes of bacteria, but harms mammalian cells only if it is introduced into a cell. Inside a mammalian cell, the proapoptotic peptide disrupts the mitochondria, as their membranes resemble those of bacteria (mitochondria are ancestrally related to bacteria). Leakage of mitochondrial membrane, in turn, is one of the main initiators of apoptosis. The efficacy of this approach has been demonstrated in mice in two diseases associated with angiogenesis, a tumor model [31] and synovial inflammation in arthritis [6]. Moreover, combining the same proapoptotic peptide with a homing peptide that binds to the blood vessels of the normal prostate yielded a compound with a different targeting specificity. Intravenous administration of this compound to male mice resulted in partial destruction of the normal prostate tissue. In a transgenic prostate cancer model (TRAMP mice), administering the prostate-targeted compound before the appearance of the tumors delayed tumor development [32]. That the same non-selective proapoptotic peptide could be used successfully for different targets by changing the homing peptide, dramatically illustrates the potential of the targeting technology.

*Specific delivery to subcellular sites.* Relatively recently, we have identified peptides that selectively recognize tumor endothelial cells and tumor cells, and are capable of delivering a drug-like payload, such as fluorescein or rhodamine, into the nucleus of these cells. One of these peptides, Lyp-1, recognizes the tumor lymphatics and tumor cells [20], whereas another peptide transports fluorescein into the nuclei of tumor blood vessel endothelial cells and tumor cells ([33]; Table 1). These peptides contain numerous basic amino acid residues, which apparently form a nuclear localization signal. They might prove to be particularly useful for delivering anti-cancer drugs that act in the nucleus. It might also be possible to develop specific targeting probes for other intracellular organelles.

*Targeted gene therapy.* Viral gene therapy vectors have been genetically modified with homing peptides and antibodies for targeting purposes. Inserting an RGD sequence into an adenovirus surface protein changes the tropism of the virus such that the virus infects cells expressing integrins [34,35]. A non-peptidic compound that binds to αvβ3 integrin has been used to target a nanoparticle-based gene therapy vector to tumor vasculature [36]. The particle consisted of a lipid micelle that carries, on its surface, the αvβ3-binding compound and a DNA-binding cationic lipid. The particle selectively delivered a mutated *Raf-1* gene to the αvβ3-expressing endothelium in tumors. A single intravenous injection of the targeted nanoparticles induced apoptosis in tumor vessels, tumor regression and prolonged survival in mice.

*Anti-angiogenic versus anti-vascular targeting.* The experimental tumor targeting studies used fast-growing mouse tumors. The blood vessels in these tumors are 'new' and might be more likely to carry angiogenesis markers, such as the αvβ3 integrin, than vessels in slow-growing tumors. Thus, the experimental tumors could be more responsive to anti-angiogenic therapies than human tumors, which grow over years and might have a greater proportion of mature vessels than the experimental tumors. Treating

tumors with well-established vessels is likely to require both anti-vascular and anti-angiogenic approaches. As blood vessels mature, they acquire a pericyte coating and associated ECM. Tumor-specific changes in these vascular elements, such as the NG2 proteoglycan in pericytes [17] and oncofetal fibronectin and altered collagen in the matrix [18,37], might make it possible to develop targeted delivery approaches for anti-vascular therapy. Partial damage to the vessels might suffice, as thrombosis of the affected vessels might be initiated that would further restrict blood flow to the tumor [38]. Targeting through the lymphatic vessels is another potential way of expanding the targeting strategy [20]. In the future, anti-vascular tumor therapies are likely to rely on drug combinations that provide a concerted attack at more than one of the vulnerabilities in tumor vasculature.

## Concluding remarks

The homing peptides isolated by phage display have shown the great diversity of blood vessels. Vascular homing peptides for a large number of tissues have already been found, which suggests that it will be possible to identify homing peptides that are specific to blood vessels in most, if not all, tissues. It is likely that additional homing peptides specific to tumor vasculature can also be found by further screening of various tumors. Uncovering the identity of the 'receptors' for homing peptides is a priority. The already known receptors (integrins, proteases and a proteoglycan) in tumor vasculature suggests that the markers of tumor vasculature discovered by phage screening will be a heterogeneous group of proteins, predominantly associated with angiogenesis. Many of these proteins are likely to prove to be functionally significant to the process of angiogenesis. In the future, vascular markers that characterize the vasculature of one tumor type, rather than angiogenesis, might also be found.

Promising results have come from the first attempts to direct drugs to tumors by using drug–peptide conjugates that home to tumor vasculature. As the receptors for the homing peptides are identified, improved versions of these peptides and their drug conjugates can be developed. As the identity of the receptors unfolds, it will also be possible to quantitatively determine the difference between the expression of these receptors within the vasculature of tumors and normal tissues. This information can be used to improve the selectivity of drug targeting by optimizing the affinity and binding valency of the homing peptides (or their mimetics). More efficient delivery of drugs, radioactive compounds and genes into tumors will undoubtedly ensue.

So far, the only pathology extensively targeted with the phage is tumors (angiogenesis). Other pathologies might also cause alterations in the resident vasculature. In fact, it is known that this happens in inflammation and ischemia. Thus, *in vivo* phage screening to target tissues affected by various diseases might prove rewarding. Another attractive possibility is screening for peptides (or proteins) that are able to cross certain barriers, such as the blood–brain barrier.

Translating homing peptide technology developed in the mouse into therapies for human disease should be possible. All of the peptides we have tested to date have recognized the equivalent human vascular sites, suggesting that the peptides already developed might be directly applicable in humans. However, it might be necessary to modify the peptides for optimal activity. The optimization might require converting the peptides into peptidomimetics or non-peptide chemistries, and will benefit from identification of the vascular receptors for the peptides. The available results demonstrate the potential of the homing peptide technology for affinity-based drug targeting. Clearly, what has been done so far represents only the beginning in exploiting its potential.

## Acknowledgement

The author's work was supported by the National Institutes of Health grants CA 82713 and Cancer Center Support Grant CA 30199, and the DAMD 17-02-1-0315 Innovator Award from the Department of Defense.

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